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Prolonged residence in the phagosomal compartment of host macrophages is essential for the establishment and maintenance of chronic infection by the brucellae in mammalian hosts. The intracellular brucellae are exposed to a variety of environmental stresses in this compartment, including nutrient deprivation and exposure to low PH and reactive oxygen intermediates. Corresondingly, a significant degree of physiologic adaptation is required in order for these bacteria to survive for prolonged periods within this niche. Studies described in this report indicate that the *Brucella* genes that contribute to this physiologic adaptation represent good targets for use in the construction of vaccine candidates. Specifically, these studies have further verified the importance of de novo purine biosynthesis for intracellular survivala and replication in host macrophages. Studies described in this report have also determined that the Brucella BacA is required for proper acylation of the libid A component of the LPS. Finally, studies employing the human monocyte-like cell line indicate that a) virulent *B. abortus* 2308 inhabits two different types of replicative vacuoles in these phagocytes, b) the isogenic hfq mutant Hfq3 is trafficked like 2308 to these vacuoles but cannot replicate therein, and c) the isogenic bacA mutant KL7 displays internalization and trafficking patterns in THP-1 cells that are different form those displayed by *B. abortus* 2308 or Hfq3.

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INTRODUCTION

Brucellosis is a serious zoonotic disease (Acha and Szyfres, 1980) that poses a potential threat to military personnel stationed in many areas of the world. These individuals are at risk either through accidental exposure to infected animals or animal products (Nicoletti, 1989) or through deliberate exposure to *Brucella* spp. as a component of biological warfare by an opposing military force (Huxsoll et al., 1987; Kaufmann et al., 1997). Unfortunately, there is currently no safe, effective brucellosis vaccine for use in humans. Prolonged survival and replication in host macrophages is a key component of disease production by *Brucella* spp. (Baldwin and Winter, 1994), and current evidence indicates that reactive oxygen intermediate (ROI)-mediated killing is the primary mechanism by which host macrophages eliminate intracellular brucellae (Jiang et al., 1993). Therefore, cellular components that contribute to the resistance of the brucellae to oxidative killing by host phagocytes represent important virulence determinants. Biochemical and genetic studies have clearly shown that bacterial stress response proteases of the high temperature requirement A (HtrA) family have the capacity to degrade oxidatively damaged proteins before they accumulate to toxic levels within cells (Davies and Lin, 1988; Johnson et al., 1991; Li et al., 1996). In performing this function, members of this particular class of periplasmic serine proteases serve as an important secondary line of defense against ROI-mediated killing. Genetic studies in numerous bacteria including *Brucella abortus* and *Brucella melitensis* have confirmed the participation of the HtrA protease in cellular defense against oxidative damage (Elzer et al., 1994; Phillips et al., 1995; Robertson et al. 1996), and further suggest that this protease contributes to the survival and replication of the brucellae in host macrophages (Elzer et al., 1996a) and their virulence in natural and experimental hosts (Edmonds et al., 1997, Elzer et al., 1994; Elzer et al. 1996a; Elzer et al. 1996b, Phillips et al. 1995; Phillips et al. 1997a, Robertson et al. 1996; Roop et al., 2001). *Salmonella* strains carrying *htrA* mutations have been used successfully as vaccines in both mice (Chatfield et al., 1992) and humans (Tacket et al., 1997), and evidence recently obtained with pregnant goats suggests that *Brucella htrA* mutants also hold promise as vaccine candidates (Phillips et al., 1997a). Unfortunately, the attenuation of the *Brucella htrA* mutants observed in the original studies in mice was limited to the early stages of infection (Elzer et al. 1996a; Phillips et al. 1995), and this residual virulence prevented their evaluation as experimental vaccines in this important model of human brucellosis. Therefore, the original objective of the proposed studies outlined in the Statement of Work for contract DAMD17-98-C-8045 was to introduce secondary mutations into *B. abortus* and *B. melitensis htrA* mutants which would enhance and stabilize the attenuation of these strains in BALB/c mice.

As detailed in previous annual reports, soon after the initiation of this project is was found that the *Brucella* mutants used in the original studies were in fact *htrA cycL* double mutants, the secondary *cycL* mutation arising from the close proximity of this gene to the 5' end of the *Brucella htrA* and the strategy employed for gene disruption (Phillips et al., 1999). Subsequent analysis of an authentic *B. abortus htrA* mutant in mice revealed no significant attenuation for this strain (Phillips and Roop, 2001). Moreover, introduction of selected tertiary mutations into the *htrA cycL* mutants did not enhance the attenuation of these strains in mice beyond their original level (Kovach and Roop, 1996). For these reasons, a revised Statement of Work was submitted for this contract and approved. The new objectives of this project focus primarily on exploring the potential of *B. abortus* and *B. melitensis hfq* and *bacA* mutants as vaccine candidates in the mouse model and further characterizing the interactions of these highly attenuated mutants with cultured murine macrophages in an effort to gain a better understanding of the basis for their attenuation. Although experimental evidence clearly establishes a generalized defect in stationary phase physiology as the basis for the extreme and stable attenuation of *Brucella hfq* mutants (Robertson and Roop, 1999), the biologic function of the *bacA* gene product was unknown at the inception of this project (LeVier et al., 2000). Thus, another focus of this project is to thoroughly evaluate the phenotypic characteristics of the *B. abortus bacA* mutant KL7 and its *B. melitensis* counterpart KL20 in an attempt to define the

biological function of the *bacA* gene product. In addition, we have recently identified other genetic loci that participate in the adaptation of the brucellae to their intracellular niche. Preliminary studies suggest that *B. abortus* mutants carrying lesions in these loci display significant attenuation in the mouse model to warrant their further examination as potential vaccine candidates.

BODY

I. *B. abortus* Tn*5* mutants demonstrating nutritional defects in vitro display differential virulence profiles in cultured murine macrophages and BALB/c mice.

Experimental evidence suggests that the brucellae encounter a considerable degree of nutrient deprivation during their long-term residence in host macrophages (Crawford et al., 1996; Robertson and Roop, 1999). Correspondingly, the ability of these organisms to maintain their metabolic versatility appears to be critical to their capacity to survive for prolonged periods in the phagosomal compartment (Köhler et al., 2002; 2003). As described in previous reports, a bank of *B. abortus* 2308 Tn*5* mutants has been constructed which display limited or no growth on solidified Gerhardt's minimal medium (GMMa), but exhibit wild type growth on Schaedler blood agar supplemented with 5% defibrinated bovine blood (SBA). A subset of these mutants that were predicted to be defective in amino acid or purine biosynthesis or the transport specific amino acids (Table 1) were evaluated for their ability to survive and replicate in cultured murine macrophages (Figure 1 and 2) and their capacity to establish and maintain chronic spleen infection in BALB/c mice (Figures 3 and 4). The *B. abortus* 2308 derivatives with Tn*5* insertions in *purE* (AR975), *purH* (AR875), *purL* (AR54) and *ilvD* (AR943) exhibited significant attenuation in both macrophages and in experimentally infected mice. The other Tn*5* mutants examined – AR93 (*trpB*::Tn*5*), AR408 (*pheA*::Tn5), AR536 (*dagA*::Tn*5*) and AR981(*leuB*::Tn*5*) – displayed essentially wild-type virulence in macrophages and mice.

The purine auxotrophy of AR54 (*purL*::Tn*5*) and AR975 (*purE*::Tn*5*), the mutants displaying the greatest attenuation in mice, was verified by the inability of these mutants to grow on GMMa unless this medium is supplemented with adenine and guanine (data not shown). A *sacB*-based counterselection strategy was used to replace the reconstruct the wild type version of the *purL* and *purE* genes in AR54 and AR975, respectively, resulting in the construction of strains designated AR54C and AR975C. Both of these latter strains grew with the same efficiency at 2308 on GMMa. AR54C and AR975C also exhibited intracellular survival and replication patterns in cultured murine macrophages equivalent to that of 2308 and wild-type virulence at four weeks post infection in experimentally-infected mice (data not shown).

II. The *B. abortus bacA* mutant KL7 displays an altered acylation pattern in the lipid A component of its lipopolysaccaride.

Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry (MS) was performed at the University of Georgia Complex Carbohydrate Research Center to analyze purified lipid A fractions from *B. abortus* 2308 and the isogenic bacA mutant KL7. Although the volatile long chain fatty acids 27-OH C28:0 and 27-O (βOmeC4:0) C28:0 and the shorter fatty acid 3-OH C14:0 are major components of the predominant lipid A species of *B. abortus* 2308 (Figure 5A), the major lipid A species of KL7 lacks these three fatty acids (Figure 5B). Unfortunately, deduced structures for these lipid A species are not available. For illustrative purposes, however, the results of MALDI-TOF MS analysis of purified lipid A from *Sinorhizobium meliloti* Rm1021 and an isogenic *bacA* mutant are shown in Figure 6, along with the deduced structure for the major lipid A species of these strains.

III. Construction and characterization of a non-polar *B. abortus rel* mutant.

The extreme attenuation of the *B. abortus rel* mutant RAB1 in cultured murine macrophages and in experimentally infected mice was described in last year's annual report. However, as also

noted in that report, the *Brucella rel* resides in an operon immediately upstream of *pyrE*, a gene whose product is required for *de novo* pyrimidine biosynthesis (Neuhard and Kelln, 1996). The mutational strategy used to construct RAB1 makes it almost certain that is strain is a *rel pyrE* double mutant. Consequently, gene replacement was used to remove 2079 bp internal to the coding region of the rel gene in *B. abortus* 2308 and replace it with the non-polar mutagenesis cassette pUC18K (Menard et al. 1993). The resulting strain was designated RAB2. Nucleotide sequence analysis of the site of the pUC18K insertion in the genome of RAB2 indicates that wild-type transcription and translation of *pyrE* should be retained in this strain, but this has not been experimental confirmed to date. Efforts are still underway to construct an isogenic *pyrE* mutant from *B. abortus* 2308. Once the *B. abortus rel* and *pyrE* mutants are in hand, they will be used to definitively evaluate the individual contributions of the corresponding gene products to virulence in the mouse model.

IV. Trafficking and replication of *Brucella* strains in the human monocyte-like cell line THP-1.

When studied by indirect immunofluorescence microscopy, the initial steps of brucellae trafficking in cultured THP-1 cells were found to be unaltered from the normal trafficking of opsonized phagocytic particles. All phagocytosed bacteria (opsonized live and dead *B. abortus* 2308, KL7 (2308 *bacA*), Hfq3 (2308 *hfq*), *Escherichia coli*) and particles (latex beads) displayed transient association with early endocytic markers. These endocytic vesicles containing bacteria or particles transition into early phagosomes through the activity of Rab7 (major regulator of phagosome and lysosomes trafficking) resulting in Lamp1 positive and acidic vesicles. Maturation into late phagosomes is indicated by the acquisition of lysosomal hydrolase Cathepsin D. It has been reported that *Brucella* avoids fusion with Cathepsin D positive vesicles in order to survive within professional phagocytes (Celli et al., 2003). Accordingly, virulent *B. abortus* phagosomes did not fuse to a high degree with Cathepsin D positive lysosomes within THP-1 cells at 2 hours post internalization and beyond. However, prior to this time, Cathepsin D did colocalize with a greater number of virulent *B. abortus* phagosomes and these percentages were similar to those phagosomes containing control particles (*E. coli*, latex beads, heat killed *Brucella*). Two scenarios could explain these observations. First, those brucellae that are found in Cathepsin D-positive compartments are destined for destruction and this fraction of the intracellular brucellae would account for those that are killed within the first 48 hours of infection in these phagocytes. In contrast, the *Brucella* that reside in compartments that avoid lysosomes then go on to survive and subsequently replicate. The second scenario is that those brucellae in phagosomes that acquire Cathepsin D are somehow able to rid the compartment of the lytic hydrolase before its damage to the bacterial cell. This could be either through the destruction of hydrolase by bacterial peptidases secreted into the lumen of the phagosome or by stimulating the cycling of Cathepsin D out of the bacterial phagosomes. Both of those scenarios can be examined by using a combination of antibodies that recognize portions of Cathepsin D (specific for either the active or inactive forms of the protein) with phagosome isolation where relative protein concentrations can be determined. Such techniques have been used to discover that an inactive immature form of Cathepsin D is delivered to phagosomes containing *Salmonella typhimurium* (Hasim et al., 2000).

It is clear that those brucellae remaining in Cathepsin D-negative, but Lamp1-positive and acidic comparments go on to replicate after 24 hours. Two types of replication patterns are observed for these bacteria. Frequently observed are two bacteria joined at the long axis where the phagosomal membrane appears to expand and elongate with the newly formed bacteria (tightly apposed phagosome). The other type of replicative vesicles observed are very large vesicles containing large numbers of brucellae where the bacteria appear to replicating free inside of the surrounding vesicle. Both the singular and large phagosomes are Lamp1-positive, but the large replicative phagosomes are less likely to be acidic. A more definitive examination of these compartments is ongoing. It also appears that the newly formed single vesicles are able

to enter the phagosomal pathway and are capable of fusing with lysosomes. This conclusion was derived from the observation that the brucellae residing in these Cathepsin D-positive compartments are expressing high levels of GFP. The GFP signal from dead *Brucella* is lost within hours after fusion with lysosomes, therefore those bacteria that remain GFP positive to a high degree are likely derived from an intracellular replication and are newly entered into the phagosomal pathway. It seems unlikely that the introduction of newly internalized brucellae could explain the occurance of the brucellae in Cathepsin D positive vesicles at these times, since brucellacidal levels of antibiotics are maintained in the cell culture medium throughout the experiment. A schematic representation of the trafficking patterns of live and dead *B. abortus* 2308 and live *B. abortus* KL7 (2308 *bacA*) and Hfq3 (2308 *hfq*) is presented in Figure 7. This figure also includes a table explaining the various functions of each of the host's vesicle trafficking components that were monitored during these studies.

V. Behavior of the attenuated *B. abortus* mutants Hfq3 (2308 *hfq*) and KL7 (2308) in THP-1 cells.

In contrast to the virulent parental 2308 strain, *B. abortus* Hfq3 displayed no net replication in the THP-1 cells (Figure 8.). Although the compartments within which Hfq3 resides do not differ from those containing 2308 (Figure 7), very few of the "replicative" compartments described in the previous section were observed in the Hfq-infected THP-1 cells. In contrast, a greater number of KL7 cells were found in Cathepsin D-positive compartments at 2 hours post infection than was observed for 2308 and Hfq3 (Figure 7), but those brucellae that survive the initial intracellular killing period appeared to be able to replicate and the "replicative" vesicles inhabited by KL7 were similar to those inhabited by 2308. Two interesting and potentially informative observations that were made with the *bacA* mutant were that this mutant showed much greater adherence to the cultured monocytic cell line (with a concomitant reduction in internalization) than did 2308 and Hfq3, and KL7 appeared to be less reactive with the *Brucella*-specific antiserum used for opsonization (as evidenced by reduced reactivity with the fluorescently labeled secondary antibody) than 2308 and Hfq3. The latter observations are consistent with changes in the hydrophobicity of the cell envelope of KL7 compared to the other two strains.

VI. Effects of IFN-γ activation on *Brucella* trafficking and replication

To address the effect of IFN-γ activation on the maturation of the *Brucella*-containing phagosome, THP-1 cells were activated with IFN-γ and the intracellular replication profiles and trafficking patterns of virulent *B. abortus* 2308 were compared in the activated and non-activated phagocytes. Although INF- γ activation resulted in greater killing of the intracelluar brucellae (Figure 9), no distinguishable difference in the percentage of intracellular *Brucella* residing in LAMP-1 positive compartments was observed between non-activated and IFN-γ activated monocytes during the first 24 hours following internalization. At 48 hours post infection, large numbers of brucellae could be observed replicating in LAMP 1-positive compartmens in the non-activated phagocytes, while very few of the IFN-γ activated cells had large numbers of replicating *Brucella*.

KEY RESEARCH ACCOMPLISHMENTS

- Identifying the *purL* and *purH* mutations as attenuating lesions in *B. abortus*, and verifying the importance of de novo purine biosynthesis for the successful adaptation of the brucellae to their intracellular niche in host macrophages.

- Determining that *Brucella purE*, *purL* and *purH* mutants display differing degrees of attenuation in the mouse model, these observations have important implications with regard to vaccine strategies aimed at balancing attenuation and immunogenicity in the host.

- Finding that genetic lesions in *B. abortus* 2308 affecting purine biosynthesis appear to have a much greater effect on virulence than those targeting amino acid biosynthesis and transport genes.

- Discovering that the lipid A moiety of the *B. abortus bacA* mutant KL7 lacks C28:0 and C14:0 or C12:0 modifications found in the parental version of this molecule, this finding providings important regarding the biological function of BacA.

- Determining that the intracellular trafficking patterns of the *B. abortus hfq* mutant Hfq3 in the human monocyte-like cell line THP-1 are similar to those displayed by the virulent parental strain 2308.

- Discovering that the *B. abortus bacA* mutant KL7 displays different internalization and intracellular trafficking patterns in THP-1 cells compared to virulent *B. abortus* 2308.

REPORTABLE OUTCOMES

Manuscripts, abstracts and presentations:

Roop, R. M. II., G. T. Robertson, G. P. Ferguson. L. E. Milford, M. E. Winkler, and G. C. Walker. 2002. Seeking a niche: putative contributions of the *hfq* and *bacA* gene products to the successful adaptation of the brucellae to their intracellular home. Vet. Microbiol. **90**:337-351 - **manuscript**

Ferguson, G. P., R. M. Roop II, and G. C. Walker. 2002. Deficiency of *Sinorhizobium meliloti bacA* mutant in alfalfa symbiosis correlates with alteration of cell envelope. J. Bacteriol. **184**:5625-5632 - **manuscript**

Fernandez-Prada, C. M., Zelazowska, E. B., M. Nikolich, T. L. Hadfield, R. M. Roop II, G. T. Robertson, and D. L. Hoover. 2003. Interactions between *Brucella melitensis* and human phagocytes: bacterial surface O-polysaccharide inhibits phagocytosis, bacterial killing and subsequent host cell apoptosis. Infect. Immun. **71**:2110-2119 – **manuscript**

Roop, R. M. II, J. Gee, G. T. Robertson, J. M. Richardson, W-L. Ng and M. E. Winkler. 2003. *Brucella* stationary phase gene expression and virulence. Ann. Rev. Microbiol. **57**: 57-76 – **manuscript**

Roop, R. M. II, B. H. Bellaire, M. W. Valderas, and J. A. Cardelli. Adaptation of the brucellae to their divergent intracellular niches. Mol. Microbiol (invited MicroReview, under revision, manuscript no. MMI-2003-01676) – **manuscript**

Bellaire, B.H., R.M. Roop II, J. Cardelli. Trafficking and residence of *Brucella abortus* inside human monocytes. 2002. Abstr. 42nd Annu. Meet. Amer. Soc. Cell Biol., Abstr. B-697 -**abstract**

Alcantara, R. B., R. D. Read, M. W. Valderas, and R. M. Roop II. 2003. *Brucella abortus* mutants with growth defects under starvation conditions are attenuated in cultured macrophages and in experimentally-infected mice. Abstr. 103rd Annu. Meet. Amer. Soc. Microbiol., Abstr. D-197 - **abstract**

Campos, E., R. Alcantara, S. Cravero, R. M. Roop II, and O. L. Rossetti. 2003. Role of *bmp18* in *Brucella abortus* virulence and maintenance of membrane integrity. Abstr. 103rd Annu. Meet. Amer. Soc. Microbiol., Abstr. D-198 – **abstract**

Alcantara, R., R. Read, and R. M. Roop II. "*Brucella abortus* mutants with growth defects under starvation conditions are attenuated in cultured macrophages and in experimentally-infected mice." 55th Annual Brucellosis Research Conference, St. Louis, MO., November, 2002 - **presentation**

Bellaire, B. H., R. M. Roop II, and J. Cardelli. "Virulent strains of *Brucella abortus* survive in a modified lysosome-like compartment within human monocytes." 55th Annual Brucellosis Research Conference, St. Louis, MO., November, 2002 - **presentation**

Nikolich, M., M. Izadjoo, R. Borschel, R. Roop, and D. Hoover. "A *Brucella melitensis purE bacA* dual mutant in mice and nonhuman primates." 55th Annual Brucellosis Research Conference, St. Louis, MO., November, 2002 - **presentation**

Alcantara, R., and **R. M. Roop II.** "*Brucella abortus* mutants with growth defects under starvation conditions are attenuated in cultured macrophages and in experimentally-infected mice." Mid-Atlantic Microbial Pathogenesis Meeting, Wintergreen, VA., February, 2003 - **presentation**

Bellaire, B. H., **R. M. Roop II**, and J. Cardelli. "Trafficking and residence of *Brucella abortus* within human monocytes." Mid-Atlantic Microbial Pathogenesis Meeting, Wintergreen, VA., February, 2003 - **presentation**

Alcantara, R., and R. M. Roop II. "*Brucella abortus* mutants with nutritional defects show attenuation in cultured macrophages and in experimentally-infected mice." 54th Annual Brucellosis Research Conference, St. Louis, MO., November, 2001 - **presentation**

R. M. Roop II. "*Brucella* spp." National Institutes of Allergy and Infectious Disease Blue Ribbon Panel on Bioterrorism and its Implications for Biomedical Research, Herndon, Virginia, October, 2002 – **presentation**

R. M. Roop II. "The *Brucella* spp.: stealth pathogens". Session 214, Biology of Selected "Select" Agents, 103rd Annual Meeting of the American Society for Microbiology, May 21, 2003 - **presentation**

R. M. Roop II. "Remarkable parallels exist between the "pathogenic" and "symbiotic" interactions of the agrobacteria, rhizobia and brucellae with their plant and animal hosts. United States-European Workshop on Challenges in Biotechnological Approaches to Disease Resistance in Plants and Animals, Washington, D.C., June 23, 2003 – **presentation**

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None

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A pre-proposal (ERMS no. 03184003) was submitted to the USAMRMC on July 3, 2003. Submission of a full scale proposal was not encouraged (notice dated July 15, 2003) based on lack of fund availability

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CONCLUSIONS

I. The isolation of multiple purine biosynthesis mutants in our genetic screen for *B. abortus* 2308 Tn*5* mutants that display nutritional defects *in vitro* and significant attenuation in the mouse model confirms the importance of *de novo* purine biosynthesis for long term residence in the phagosomal compartment of host macrophages.

The experimental findings reported here confirm and extend those of other groups (Borschel et al, 1999; Cheville et al. 1996; Crawford et al., 1996; Drazek et al., 1995; Köhler et al. 2002) and verify that *de novo* purine biosynthesis is essential for long-term residence of the brucellae in the phagosomal compartment of host macrophages (Alcantara et al., 2003). They also provide further evidence that *Brucella* purine auxotrophs represent attractive vaccine candidates. Moreover, the differing degrees of attenuation displayed by the *B. abortus purE*, *purH* and *purL* mutants in mice suggests that it will be possible to strategically target individual genes in the purine biosynthetic pathways and engineer vaccine candidates displaying the desired balance of attenuation and immunogenicity.

Purine analogs have been evaluated as potential chemotherapeutic against *Mycobacterium tuberculosis* (Bakkestuen et al., 2000; Scozzafava et al. 2001). Based on the critical nature of *de novo* purine biosynthesis for the successful survival of the brucellae in their intracellular niche, these purine analogs may also be effective as brucellacidal agents *in vivo*. Such a finding would be particularly important with regard to the treatment of human brucellosis since this disease is notoriously difficult to cure with the currently used chemotherapeutic regimens (Young, 2000).

II. Mutations affecting *Brucella abortus* amino acid biosynthesis and transport genes have less impact on virulence in the mouse model than those affecting purine biosynthesis.

Although a limited number of mutants were evaluated, the experimental findings presented in this report suggest that *Brucella* genes involved in the biosynthesis and transport of amino acids are less attractive as targets for mutagenesis aimed at the construction of vaccine candidates than are those that are required for *de novo* purine biosynthesis. For instance, with the exception of the *ilvD* mutant AR943, the remainder of the *B. abortus* strains with Tn*5* insertions in amino acid biosynthesis genes displayed little, if any, attenuation in murine macrophages or in experimentally infected mice. This suggests that *B. abortus* can tolerate mutations that affect the biosynthesis of individual amino acids (e.g. *leuB*, *pheA* and *trpB*) during its prolonged residence in the phagosomal compartment, but cannot overcome the effects of mutations that affect the synthesis of multiple amino acids. Such a relationship is certainly supported by a comparison of the *in vivo* phenotypes of *Brucella aroC*, *pheA* and *trpB* mutants for example. While *B. suis aroC* mutants are highly attenuated in mice (Foulongne et al. 2001), *B. abortus pheA* and *trpB* mutants are not. The *aroC* product is involved in the production of chorismate, a common precursor required for the biosynthesis of all three of the aromatic amino acids (phenylalanine, tryptophan and tyrosine) (Pittard 1996). The *pheA* and *trpB* products, in contrast, participate exclusively in the conversion of chorismate to phenylalanine and tryptophan, respectively. A similar relationship is observed when the virulence properties of the *B. abortus ilvD* mutant AR943 and *leuB* mutant AR981 are compared. The *ilvD* gene product, dihydroxy-acid dehydratase, participates in the biosynthesis of all three of the branched chain amino acids (isoleucine, leucine and valine) (Umbarger 1996), while the *leuB* product functions exclusively in the biosynthesis of leucine. Accordingly, the *ilvD* mutant AR943 displays significant

attenuation in both macrophages and mice, while the isogenic *leuB* mutant exhibits wild-type virulence. One possible explanation for the minimal impact of the mutations affecting genes involved in the synthesis of individual amino acids on virulence in *B. abortus* is the fact that the genome sequences of *B. melitensis* 16M (delVecchio et al., 2002) and *B. suis* 1330 (Paulsen et al., 2002) indicate that the brucellae possess an unusually large number of amino acid transport systems compared to other prokaryotes. These transport systems may allow the brucellae to compensate for the inability to synthesize single amino acids while residing in the phagosomal compartment, but they may not be able to overcome the effects of mutations that affect the synthesis of multiple amino acids. The presence of multiple amino acid transport systems may also explain why the *dagA* mutation in AR536 has little effect on the virulence of this strain in mice.

It is important to note that the wild type virulence displayed by the *B. abortus leuB* mutant AR981 in murine macrophages and in mice appears to conflict with the recent report of Köhler et al (2002) who found that derivatives of *B. suis* 1330 with Tn*5* insertions in *leuA* and *leuC* were attenuated in the human macrophage-like THP-1 cell line. Whether these findings arise from differences in the metabolic makeup and nutritional requirements of the parental *B. abortus* 2308 and *B. suis* 1330 strains, or a differences in leucine availability in the phagosomal compartments of human and murine macrophages is unknown.

III The *Brucella* BacA participates in proper acylation of the lipid A component of the lipopolysaccharide.

The B. abortus *bacA* mutant KL7 exhibits significant attenuation in cultured murine macrophages and cannot maintain chronic spleen infection in experimentally infected BALB/c mice (LeVier et al., 2000; Roop et al. 2002). The *bacA* gene was originally identified in a close phylogenetic relative of the brucellae, *Sinorhizobium meliloti*, during a genetic screen for tranposon mutants that were unable to maintain symbiosis with their plant hosts (Glazebrook et al., 1993). Sequence analysis suggested that BacA is a transporter that spans the cytoplasmic membrane, but what it transported and in what direction, were unknown. Phenotypic evaluation of *S. meliloti* and *B. abortus bacA* mutants suggested that BacA played an important role in maintaining the integrity of the cell envelopes in these bacteria (Ichige and Walker 1997, Ferguson et al., 2002). The first real breakthrough with regard to defining BacA function came from the observation that the fatty acid compositions of LPS preparations from *S. meliloti* and *B. abortus bacA* mutants differed from those of their parental strains. A more detailed analysis of these differences has revealed that the major difference lies in the absence of a volatile long chain (C28) fatty acid from the lipid A of the *S. meliloti* and *B. abortus bacA* mutants that is present in the lipid A of the parental strains. These results were particularly interesting and potentially enlightening in view of the fact that BLAST analysis of BacA suggested that this protein shares significant amino acid homology with a family of eukaryotic peroxisomal membrane proteins that includes human Adrenoleukodystrophy protein (hALDP) (Kemp et al. 2001). These proteins are involved in β–oxidation of volatile long chain fatty acids (VLCFAs) by peroxisomes (Braiterman et al. 1999) and there is experimental evidence that the *Saccharomyces cerevesiae* hALDP homolog PXA1/PXA2 transports VLCFAs across the peroxisomal membranes (Verluer et al. 1997). The experimental findings presented here indicate that BacA is required for wild-type acylation of the lipid A moiety of the *S. meliloti* and *B. abortus* LPS. More importantly, the phenotypic properties of *S. meliloti* and *B. abortus bacA* mutants suggest that the presence of the specific VLCFAs is important for the maintenance of cell envelope integrity, especially when these bacteria are subjected to environmental stresses (Ferguson et al., 2002; Roop et al. 2002). Further study will be required to more precisely defined the role of BacA in determining the VLCFA composition of the lipid A. It will also be important to determine whether BacA participates in a constitutively produced biosynthetic pathway, or whether it works together with other enzymes to modify the fatty acid composition of the lipid in response to changing environmental conditions.

Despite not knowing its precise function, knowing that the presence of BacA is required for proper long chain fatty acid composition of the lipid A in *S. meliloti* and *B. abortus* explains many of the phenotypes that were originally observed in the *bacA* mutants (Ichige and Walker 197; Ferguson et al, 2002). For example, it has been postulated that the length of the C28:0 fatty acids might allow them to stabilize the outer membrane when they are present in the lipid A of *Rhizobium leguminosarum* (Kannenberg and Carlson, 2001) and a role for BacA in the insertion of these long chain fatty acids into the lipid A is certainly consisent with the observation that *S. meliloti* and *B. abortus bacA* mutants display increased sensitivity to detergents and other environmental stresses that perturb cell envelope integrity compared to their parental strains. *S. meliloti* and *B. abortus bacA* mutants are also more sensitive to low pH than their parent strains (Ichige and Walker, 1997; Ferguson et al, 2002; Roop et al. 2002). With regard to a possible role for BacA in lipid A modification, this phenotype is particuarly interesting because it has been proposed that the rhizobia increase the proportion of long chain fatty acids in their lipid A during their conversion to the bacteroid state as a means of resisting the acidic conditions encountered in the symbiosome of the plant (Kannenberg et al. 2001).

Based on what we presently know about BacA function, it is tempting to speculate that this protein plays a role in the insertion of long chain fatty acids into the lipidA of the *B. abortus* and that this modification results in an increased resistance of the cell envelope to the acidic conditions encountered in the phagosomal compartment (Phillips et al. 1997b; Porte et al., 1999). This change in lipid A acylation pattern may also aid the brucellae in resisting the effects of antimicrobial peptides encountered the extracellular environment in the host as well as during their encounters with neutrophils (Guo et al. 1998). It is also possible that the change in the fatty acid composition of the lipid A in the *B. abortus bacA* mutant affects the immunomodulatory properties of this molecule. The lipid A molecules of most Gram-negative bacteria bind to Toll-like receptor 4 on host cells and elicit a strong innate immune response (Raetz and Whitfield, 2002). In contrast, it is well documented that the *Brucella* lipid A is less active in this regard than most other lipid A molecules (Rasool et al., 1992), and indeed it has been proposed that this property may allow the brucellae to evade the induction of a full blown innate immune response in the host during the initial stages of the infectious process. With this in mind, it is conceivable that the altered form of lipid A in the *B. abortus bacA* mutant KL7 elicits a stronger and more effective innate immune response in the host than the parental strain does, eventually leading to accelerated clearance of the mutant from the host. To address this possibility, studies of the interactions of lipid A preparations from *B. abortus* 2308 and the isogenic *bacA* mutant KL7 are planned.

IV. Construction of a *B. abortus* non-polar *rel* mutant will facilitate an evaluation of the importance of the stringent response to successful adaptation to the nutritional deprivation encountered in the phagosomal compartment.

The stringent response is a global genetic and physiologic response that allows prokaryotes to make optimal use of available nutrients during periods of intense nutrient deprivation (Cashel and Rudd, 1987). Our initial findings with the *B. abortus rel* mutant RAB1 would also seem to suggest that the stringent response plays a critical role in the successful adaptation of the brucellae to the environmental conditions encountered during their long term residence in the phagosomal compartment. As outlined in last year's report, however, interpretation of the results is complicated by the fact that RAB1 is probably a *rel pyrE* double mutant, based on the genetic organization of these loci and the mutational strategy employed to construct RAB1. The *pyrE* mutation by itself should lead to pyrimidine auxotrophy (Neuhard and Kelln, 1996), which would make accurately defining the independent contributions of *rel* and *pyrE* mutations to the RAB1 phenotype very difficult, if not impossible, from a genetic standpoint. Construction of the *B. abortus* non-polar *rel* mutant RAB2 provides us with a means of definitively evaluating the contribution of the stringent response to virulence, and a thorough examination of the in vitro and in vivo properties of this mutant is planned.

V. The intracellular trafficking patterns of the B. abortus bacA mutant KL7 and the hfq mutant Hfq3 in the human monocyte-like cell line THP-1 help define the basis for the attenuation of these strains.

The results presented in this and last year's annual report demonstrate the utility of the human monocyte-like cell line THP-1 as a model for studying the intracellular trafficking patterns of *Brucella* in host macrophages. Specifically, the interactions of the virulent *B. abortus* 2308 strain and the isogenic bacA and hfq mutants with this cell mirror those observed previously with primary cultures of human monocytes and resident peritoneal macrophages obtained from mice. An added advantage of using this cell line is that it can be genetically or biochemically manipulated in a more predictable and reproducible fashion in an effort to evaluate the contributions of specific host cell components to intracellular trafficking of the brucellae than is possible with primary cell cultures.

The results of studies of the trafficking studies in the THP-1 cells has yielded important findings to date that will begin to help us better understand the mechanisms employed by the brucellae to maintain long term residence in the phagosomal compartment of host macrophages. First of all, it appears that virulent *B. abortus* 2308 can replicate in two apparently different types of replicative compartments in these phagocytes. One of these compartments resembles what has been referred to in the literature as a "tight" phagosome, the other resembles a "spacious" phagosome. The second important observation is that while the hfq mutant Hfq3 is trafficked in the same pattern as the virulent 2308, but does not survive in these intracellular compartments, the *bacA* mutant KL7 undergoes a different intracelluar trafficking pattern in the THP-1 cells. These findings are consistent with our proposition that the basis for the attenuation of the hfq mutant is its inability to make the physiologic shift required for prolonged residence in the phagosomal compartment as opposed to an inability of this mutant to produce cell components that directly influence the cell biology of the host cell. It is also not surprising that the altered biochemical and/or biophysical properties of the cell envelope of the *bacA* mutant results in a reduced efficiency of opsonization of this mutant by the *Brucella*-specific serum. Inefficient opsonization could be leading to a significant proportion of the KL7 cells being taken up by the THP-1 cells via non-opsonic pathways, and entry is this manner could result in altered intracellular trafficking patterns compared to *Brucella* cells that are taken up by opsonic pathways. It is also conceivable, however, that the altered envelope properties of the *bacA* mutant have a direct effect on the interactions of the brucellae with the phagosomal membrane and thereby alter the interaction of this membrane with other host cell compartments, which would obviously affect maturation of the *Brucella*-containing compartment. The last notable result obtained from these studies was the observation that INF-γ activation of the THP-1 cells did not seem to influence the trafficking patterns of virulent *B. abortus* 2308 in the phagocytes, but rather the activated macrophages appeared to be better able to kill the intracelluar brucellae regardless of whether or not they were residing in the "replicative" compartments described above.

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APPENDIX A. FIGURES AND TABLES FOR THE ANNUAL REPORT.

Table 1. *B. abortus* Tn*5* mutants evaluated in cultured murine macrophages and experimentally infected mice.

Mutant	Tn-*5* disrupted gene	Proposed function of disrupted gene product	Accession Number[a]
AR54	*purL*	purine biosynthesis	BMEI1124
AR93	*trpB*	tryptophan biosynthesis	BMEI2018
AR408	*pheA*	phenylalanine biosynthesis	BMEI1905
AR536	*dagA*	glycine, alanine and serine transport	BMEII0783
AR875	*purH*	purine biosynthesis	BMEI0233
AR943	*ilvD*	branched chain amino amino acid biosynthesis	BMEI1848
AR975	*purE*	purine biosynthesis	BMEI0296
AR981	*leuB*	leucine biosynthesis	BMEII0404

[a]Open reading frame designation in the *B. melitensis* genome.

Fig. 1. Intracellular survival and replication of *B. abortus* strains 2308 (•), AR54 [2308 *purL*::Tn5] (□), AR875 [2308 *purH*::Tn*5*] (×), and AR975 [2308 *purE*::Tn*5*] (▲) in cultured murine resident peritoneal macrophages. Results are expressed as percent survival following phagocytosis plus or minus the standard deviation.
* Denotes significant difference as determined by the Student's *t*-test ($p < 0.05$)

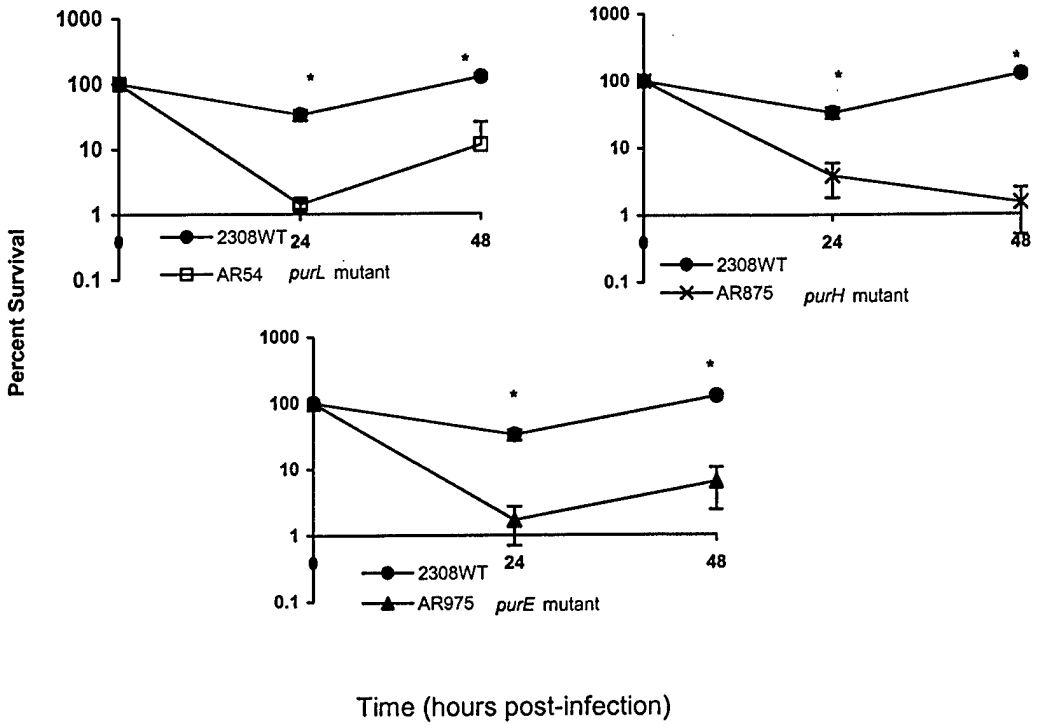


Fig. 2. Intracellular survival and replication of *B. abortus* strains 2308 (●), AR93 [2308 *trpB*::Tn*5*] (■), AR408 [2308 *pheA*::Tn*5*] (♦), AR536 [2308 *dagA*::Tn*5*] (▲), AR943 [2308 *ilvD*::Tn*5*] (×), and AR981 [2308 *leuB*::Tn*5*] (◇) in cultured murine resident peritoneal macrophages. Results are expressed as percent survival following phagocytosis plus or minus the standard deviation.
* Denotes a significant difference between 2308 and the mutant as determined by the Student's *t*-test ($p < 0.05$).

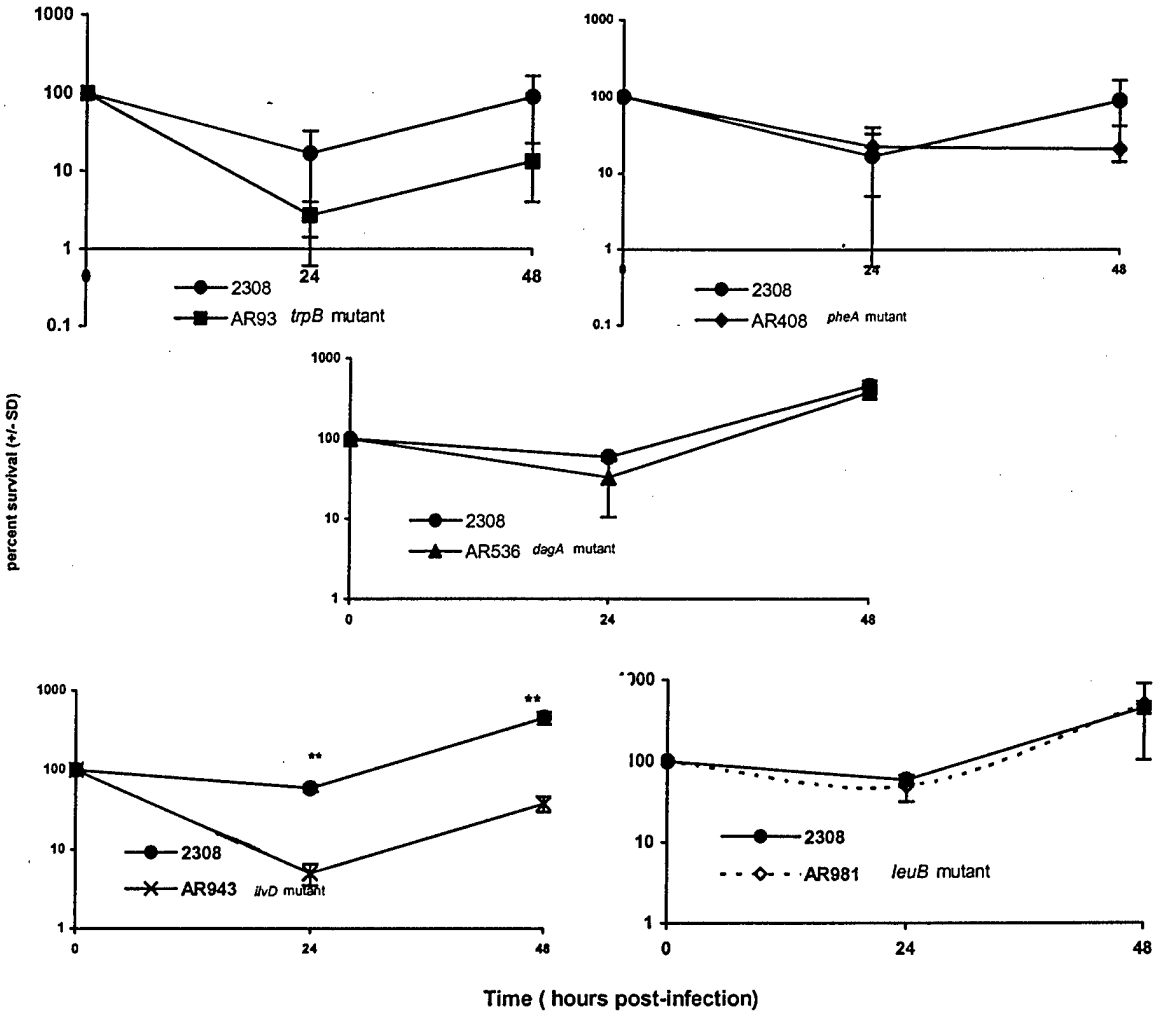


Fig. 3. Spleen colonization profiles of *B. abortus* strains 2308 (●), AR54 [2308 *purL*::Tn*5*] (□), AR875 [2308 *purH*::Tn*5*] (×), and AR975 [2308 *purE*::Tn*5*] (▲) in experimentally infected BALB/c mice. Results are expressed as total colony-forming units per spleen plus or minus the standard deviation.
** Denotes a significant difference between 2308 and the mutant as determined by the Student's *t*-test ($p < 0.01$).

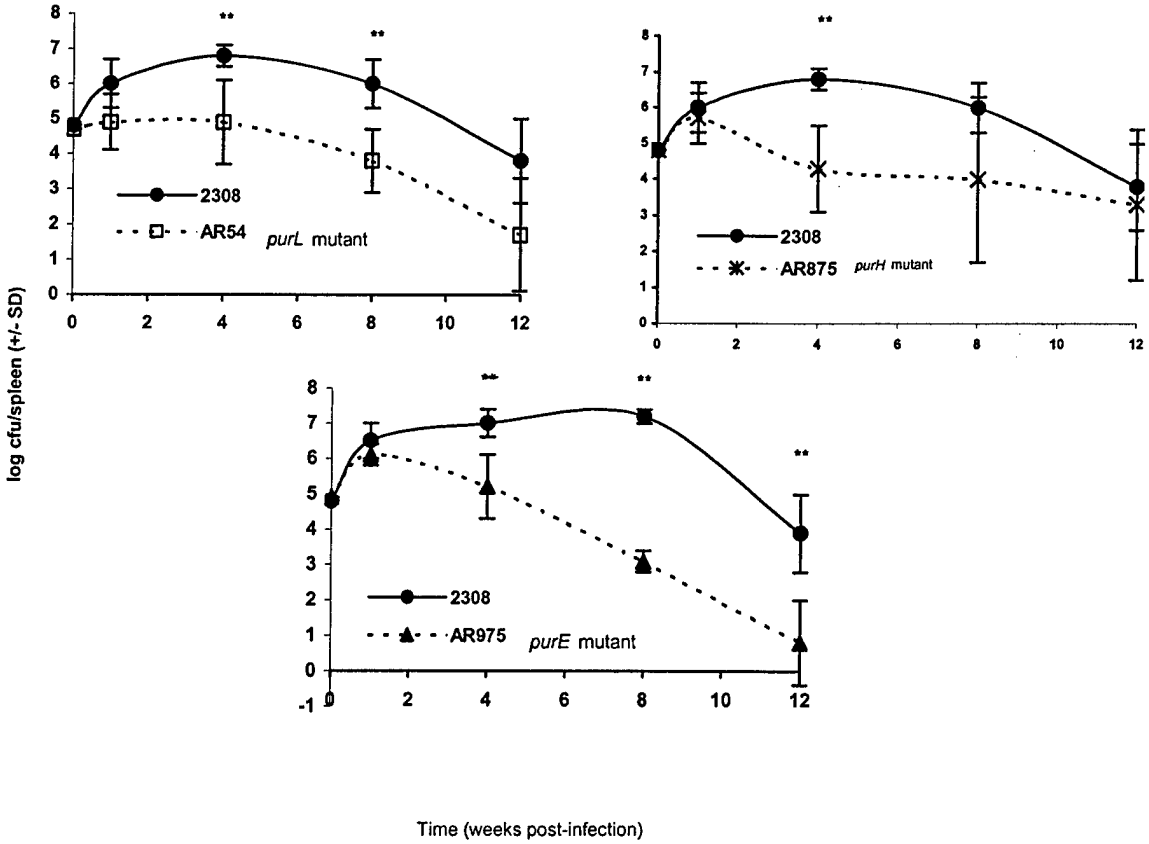


Fig. 4. Spleen colonization profiles of *B. abortus* strains 2308 (●), AR93 [2308 *trpB*::Tn*5*] (■), AR408 [2308 *pheA*::Tn*5*] (♦), AR536 [2308 *dagA*::Tn*5*] (▲), AR943 [2308 *ilvD*::Tn*5*] (×), and AR981 [2308 *leuB*::Tn*5*] (◇) in experimentally infected BALB/c mice. Results are expressed as total colony-forming units per spleen plus or minus the standard deviation.
* Denotes a significant difference between 2308 and the mutant as determined by the Student's *t*-test ($p < 0.05$).
** Denotes a significant difference between 2308 and the mutant as determined by the Student's *t*-test ($p<0.01$).

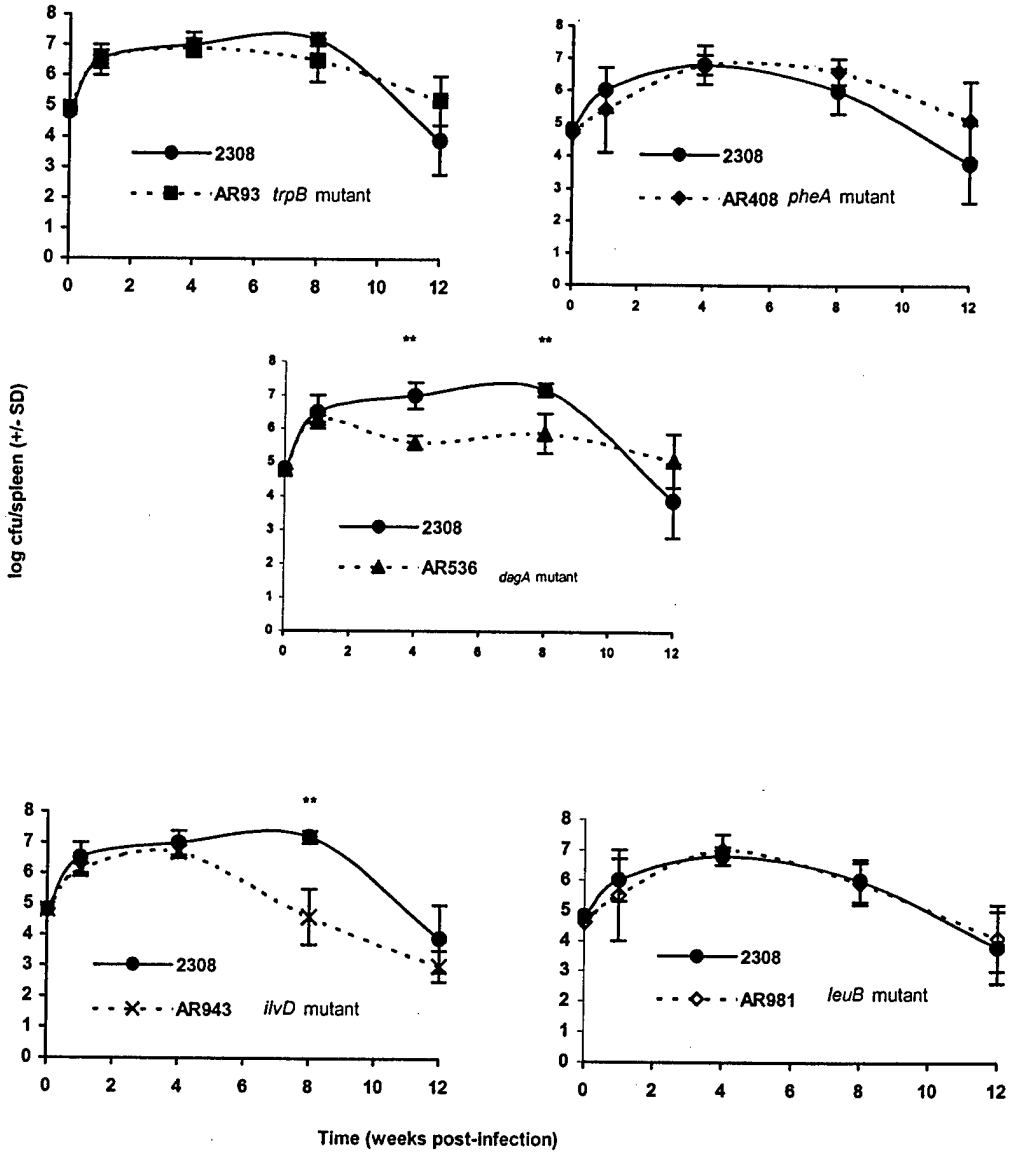


Figure 5. MALDI-TOF MS analysis of purified lipid A preparations from (A) *B. abortus* 2308 and (B) *B. abortus* KL7 (2308 *bacA*). The lipid A species represented by peak 2116 is enriched in the 27-OH C28:0 and 3-OH C14:0 volatile long chain fatty acids, while that represented by peak 2201 is enriched in 27-O(β)MeC4:0) C28:0 and 3-OH C14:0. The lipid A species represented by peak 1467 lacks all three of these VLCFAs.

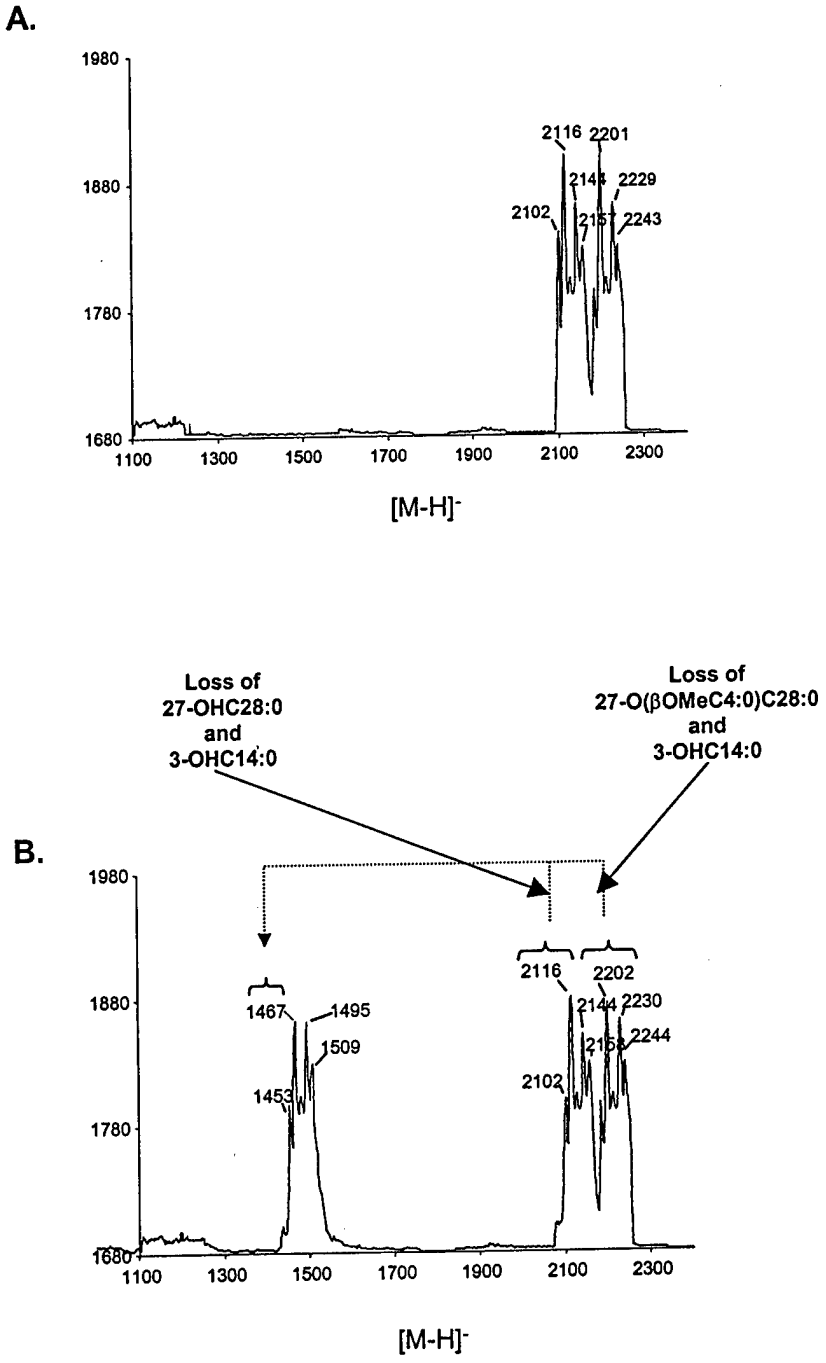
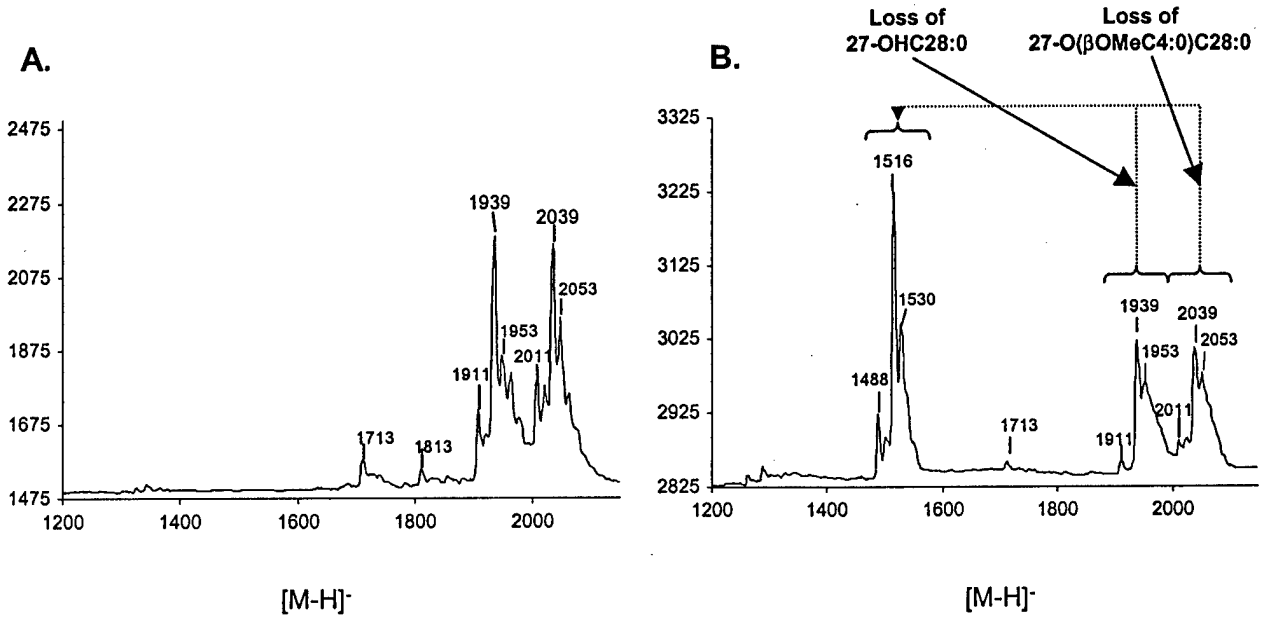
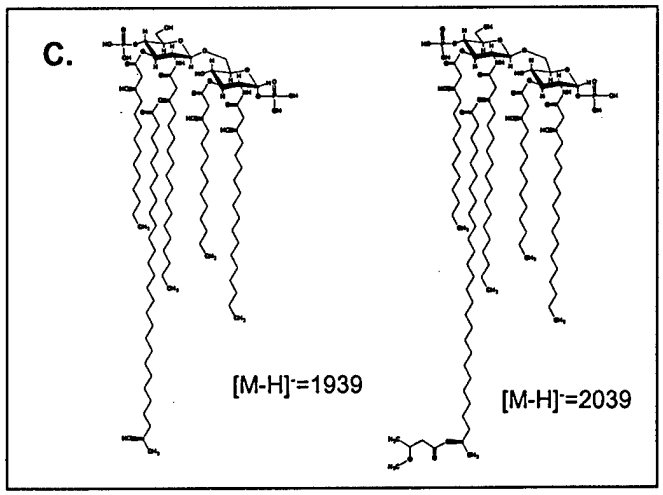


Figure 6. MALDI-TOF MS analysis of purified lipid A preparations from (A) *S. meliloti* Rm1021 and (B) *S. meliloti* Rm1021 *bacA* and the deduced structures of the major lipid A species of (C) *S. meliloti* Rm1021 and (D) *S. meliloti* Rm1021 *bacA*. The lipid A species represented by peaks 1939 and 2039 are enriched in the 27-OH C28:0 and 27(βOmeC4:0) C28:0 volatile long chain fatty acids, respectively, while peak that represented by peak 1516 lacks both of these VLCFAs.





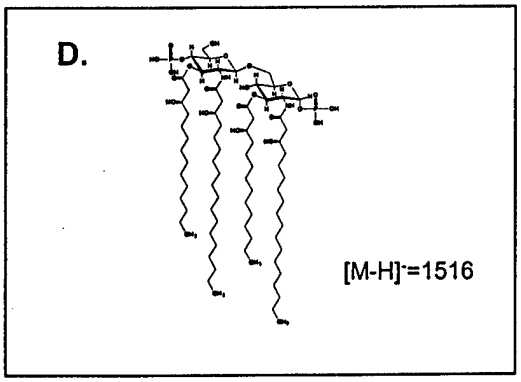
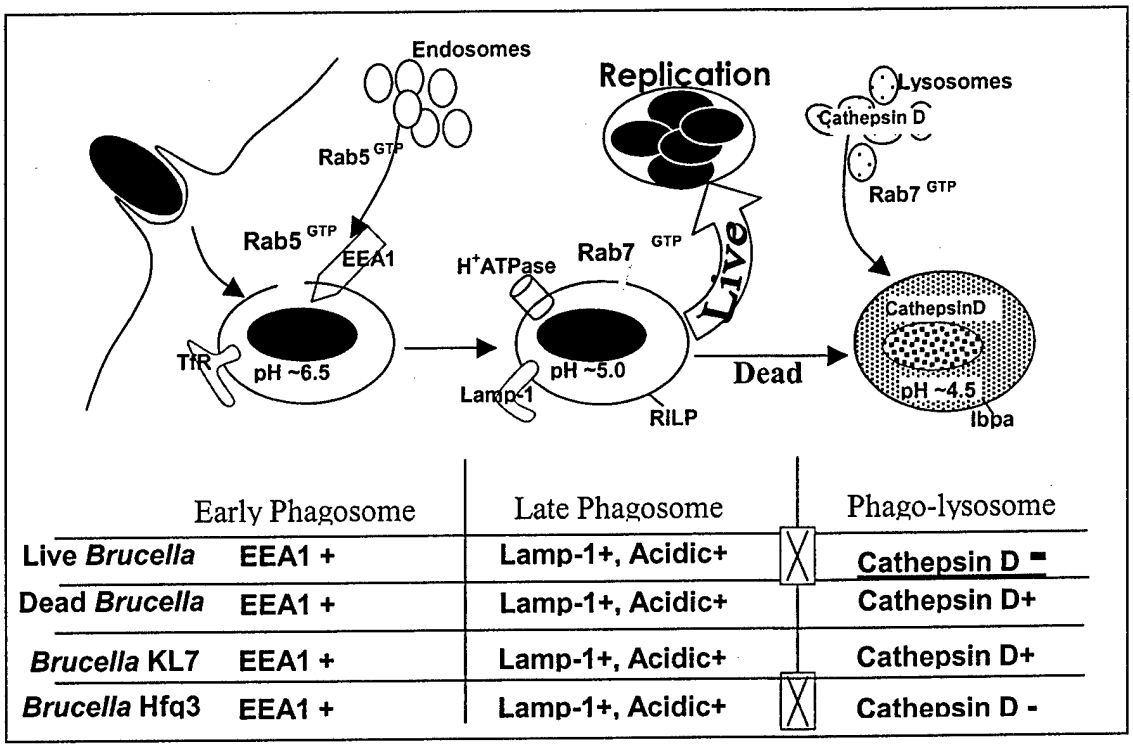


Figure 7. Schematic representation of the intracellular trafficking patterns displayed by live and dead *B. abortus* 2308 and live *B. abortus* Hfq3 (2308 *hfq*) and KL7 (2308 *bacA*) in the human monocytic cell line THP-1. The table lists the representative cell compartment-specific marker used for the trafficking studies and explains their relevance.



Marker	Localization	Notes
Trasferrin Receptor	Early endosomes/ Recycling endosomes	Uptake of Transferrin
Rab5	Early endosome/phagosomes	GTPase recruited effectors; Rabaptin5, Rabex-5 Rabphilin, EEA1
EEA1	Early endosomes	Recruits SNARE's to early endosome
Ca^2 independent Mannose-6-Phosphate Receptor	TGN and Late endosomes	Formation and targeting of lysosomes
LAMP-1	Late endosomes /early phagosomes	Present on early phagosomes
V-ATPase	Late endosomes early phagosomes	Acidifies lumen of vesicles prior to fusion with lysosomes
Cathepsin D	Lysosomes and phagolysosomes	Lysosomal hydrolase
Rab7	Phagosomes	GTPase, likely regulates phagosome maturation and fusion with lysosomes
Lysobisphosphotydic acid	Late phagosomes/phagolysosomes	Specific for inner membrane of late phagosomes
RILP	Late endosomes/ phagosomes	Co-localizes with Lamp-1 Compensates for the loss of Rab7h
lbpA	Phago/lysosomes Autophagosomes	Marker for vesicles where degredation takes place. May protect lipids from digestion.

Figure 8. Intracellular survival and replication of *B. abortus* 2308, KL7 (2308 *bacA*) and Hfq3 (2308 *hfq*) in the human monocytic THP-1 cell line.

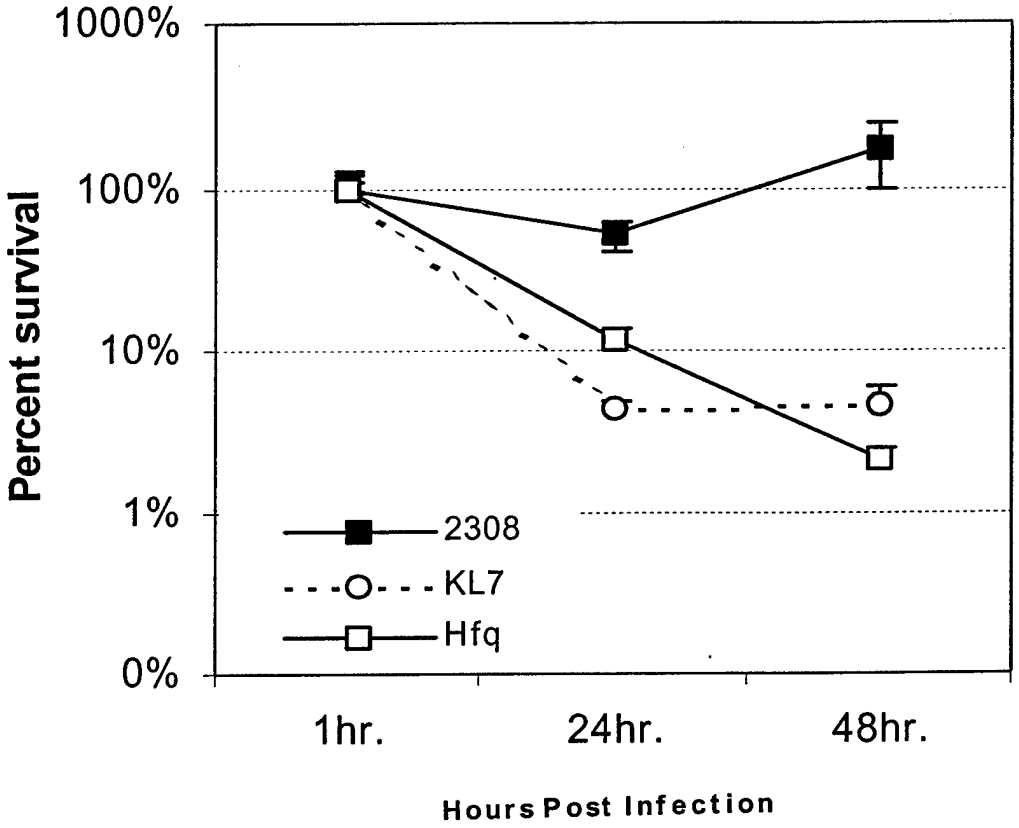


Figure 9. Intracellular survival and replication of *B. abortus* 2308 in non-activated THP-1 cells (■) and THP-1 cells activated with 50 (Δ) and 200 (×) U/ml IFN-γ.